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Isolation and Identification of *Bacillus Subtilis* and *Pseudomonas Fluorescens* from Wheat Rhizosphere and Their Use as Biocontrol Agents

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ABSTRACT

Some bacteria may be used as biocontrol agents against fungal pathogens. Biocontrol agents are environment friendly and cost effective for controlling different plant pathogens. Fungal plant pathogens cause detrimental effects on plants causing diseases and yield loss. The bacterial strains *Pseudomonas fluorescens* and *Bacillus subtilis* live abundantly in rhizospheric soil and have antagonistic activity against other organisms. The objective of present study was to isolate and identify the *Pseudomonas fluorescens* and *Bacillus subtilis* from rhizospheric soil of *Triticum aestivum* and their use as biocontrol agents against *Fusarium oxysporum and Botrytis cinerea*. The culture method, microscopic analysis and biochemical methods were used for initially screening of bacteria strain found in rhizospheric soil of *Triticum aestivum.* The biochemical and molecular tests resulted in the identification of *Pseudomonas fluorescens* and *Bacillus* from rhizospheric soil of *Triticum aestivum*. 16s rRNA sequence analysis confirmed the presence of *Bacillus subtilis and Pseudomonas fluorescens.* Biocontrol activities of *Pseudomonas fluorescens* and *Bacillus* were visualized on potato dextrose agar "PDA" + 0.5% yeast extract plate. *Bacillus subtilis showed* the maximum biocontrol activity against *Fusarium oxysporum and Botrytis cinerea*. *Pseudomonas fluorescens* showed activity against *Fusarium oxysporum* but did not show any activity against *Botrytis cinerea. Bacillus subtilis and Pseudomonas fluorescens* inhibited the development of plant pathogenic fungi *Fusarium oxysporum and Botrytis cinerea. Bacillus subtilis and Pseudomonas fluorescens* may be used as biofertilizer and biopesticides.

INTRODUCTION

Agriculture is a primary food source provider, but plant diseases are a significant risk to human societies related to agriculture. Fungal diseases are the main threat to plants. Plant Phyto pathogens have reduced the 15-20% crop yield worldwide. The loss of crops due to plant pathogens in agriculture significantly threatens the economy. Different types of fungal diseases which affect crops are(Rizzo *et al*., 2021)

Insects or diseases can be controlled with biological pesticides, also known as biopesticides, biological controls, or biocontrols. Agriculture is the sector where these pesticides are most frequently employed. Most of the time, biopesticides use interactions in the food chain between natural predators, parasitoids, fungi, or nematodes that feed on a particular pest.

Pesticides and insecticides are used to protect plants against fungal pathogens (*Fusarium oxysporum, Botrytis cinerea)*. Pesticides and insecticides are not environment friendly as these chemicals brings toxic compounds to the soil and plants. These chemicals reduce the natural value of

food. Biocontrol is important natural strategy to control pathogen.

In biocontrol we use bacteria, fungus, and viral species. Biocontrol of pathogen microorganisms didn't spread any toxic chemicals to the soil as this method is natural and environment friendly. Another advantage of bio controlling plant pathogens is the cost effectiveness. Bacterial species such as *Pseudomonas fluorescens and Bacillus subtilis* may be used as biocontrol agent against selected plant pathogens (*Fusarium oxysporium, Botrytis cinerea*). So the objectives of this study was "To isolate and identify *Bacillus subtilis* and *Pseudomonas fluorescens* from rhizospheric soil of *Triticum aestivum"* and also to investigate "To assess the biocontrol activity of *Bacillus subtilis* and *Pseudomonas fluorescens* against selected plants pathogens (*Fusarium oxysporum, Botrytis cinerea*)".

MATERIAL AND METHODS

Soil Sampling: Ten samples of soil were collected from *Triticum aestivum* rhizospheric soil at Havelian, District Abbottabad.

Bacterial isolates: Two strains of bacteria were isolated from the rhizospheric soil of wheat. i.e.

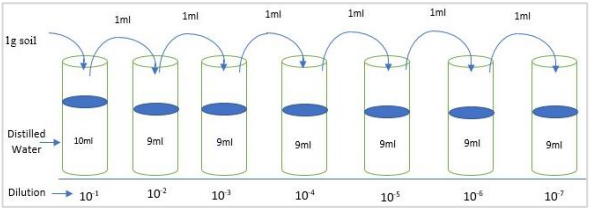
1. *Pseudomonas fluorescens*
2. *Bacillus subtilis*

Serial dilution of soil sample: 1g of rhizospheric soil was weighed and shifted to a test tube containing 10ml of distilled water and vortexed. 1 ml of diluted sample was pipetted, moved to another tube with 9ml of distilled water, and vortexed. For the 3rd, 4th, 5th, 6th, and 7th tubes with 9ml distilled water, applied the same procedure as mentioned above.

1ml from the 3rd, 5th, and 7th dilution was pipetted and poured on three nutrient agar plates from each of the 10 soil samples. These plates were incubated at 28ºC for (24 hr) for growth.

Figure 1

Serial dilution of soil sample



Specific selective media: Mannitol egg Yolk Polymyxin agar (MYP Agar) was used as selective media to isolate and identify *Bacillus subtilis. In contrast,* Cetrimide agar was used as particular media to isolate and identify *Pseudomonas fluorescens.*

Identification of isolated bacteria

Gram staining: The prominent colonies were picked with a sterile loop and placed on a sterile slide having 1 drop of distilled water and fixed by moving the slide above the spirit lamp. Gram staining was performed to check the bacteria, whether Gram positive or negative, morphology, rods, spherical, or shape. Four steps of gram staining:

a. apply crystal violet dye on a slide for 1 min and wash
b. use 1 drop of iodine for 1 min and wash
c. apply 1 drop of decolorizer for 10 sec and wash
d. apply 1 drop of safranin for 1 min and wash

The slide was dried and then examined under the microscope. The results were noted, and these bacteria were cultured on different plates to make pure culture for identification through various biochemical tests.

Preparation of Stock Sample: 50ml of 100% glycerol was mixed with 50ml of distilled water and autoclaved. 500ul of overnight grown bacterial culture were combined with 500ul of 50% glycerol and stored at -80°C.

Biochemical Tests: VP test, Methyl red test, Citrate test, Catalase test, Indole test were performed to identify pathogens

Molecular Identification of Isolated Bacteria: For the identification of bacterial strains, molecular identification is required.

DNA Extraction

DNA was extracted through the Qiagen kit method. The Bacterial isolated strains, which show good activity against plant fungal pathogens (*Fusarium oxysporum and Botrytis cinerea),* are cultured overnight for new growth. Glass bead tubes were used to lyse cultured bacteria before solubilizing proteins and nucleic acids and combining them with a binding solution.

Table 1

Primers Name	Sequence 5'- 3'	Primers Length	Product Size	Target region	References
16S Pse F	GACGGGTGAGTAATGCCTA	21	618bp	16s rRNA	(Husseyn, Nalbandyan *et al*. 2017)
16S Pse R	CACTGGTGTTCCTTCCTATA	20	618bp	16s rRNA	(Husseyn, Nalbandyan *et al*. 2017)
16S 907 R	CCGTCAATTCMTTTRAGTTT	20	592bp	16s rRNA	Lane *et al*., 1985
16S 337 F	GACTCCTACGGGAGGCWGCAG	21	592bp	16s rRNA	Lane *et al*., 1985

The flow-through was paired with a solution to bind the total RNA on a second spin column while the DNA bound to the first spin column. Protein was caught at the last flow-through column. The immobilized analyte was then eluted, and each spin column was washed.

PCR Amplification:

Polymerase Chain Reaction Conditions: The mixture for PCR reaction included 10 µL of 1x PCR buffer from Thermo Fisher Scientific, 2µ L of 10 mM dNTP mix, 1 µL of each primer (forward and reverse) in 10 mM concentration from IDT Australia (Institute of Drug Technology Limited), 1 µL of Platinum Taq polymerase (one unit per reaction), 1.5 mM of Magnesium chloride, and 10 ng/µL of DNA. With sterile MilliQ water, the reaction mixture was diluted to a volume of 50µ L. Following were the conditions used to conduct the PCR reaction in a Thermo Cycler:

Table 2

PCR Conditions for 16S PSE

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	4min	35 Cycles
Denaturation	95°C	30sec	
Annealing	50°C	30sec	
Initial Extension	72°C	30sec	
Final Extension	72°C	5min	
Infinity	4°C	∞	

PCR conditions for 16S RRNA (16S 907)

Table 6

PCR Conditions for 16S 907

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	4min	35 Cycles
Denaturation	95°C	30sec	
Annealing	57.7°C	30sec	
Initial Extension	72°C	30sec	
Final Extension	72°C	5min	
Infinity	4°C	∞	

Gel Electrophoresis and Sequencing: To verify the presence of the PCR product, the gel documentation system was used to view the PCR products under a 1% agarose gel stained with RedSafe Nucleic Acid Staining Solution (approximately 592 bp and 618 bp). Qiagen's QIAquick PCR Purification Kit was used to purify the PCR products. Nanodrop was used to quantify them before sending them to Alpha Genomics for sequencing at a concentration of 20 g/L. Using the NCBI BLAST blasting suite, the FASTA sequences of all the cultures were analyzed (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Additionally, sequences with high identity and similarity of 95 and 98% were used as the standards for classifying the DNA sequences (Barghouthi, 2011)

Antifungal Activity Testing Procedure

Fungal pathogenic strains: *Fusarium oxysporum* and *Botrytis cinerea* were used for demonstration of antifungal activity.

Media; PDA+ 0.5% Yeast Extract plate. (Yeast Extract Medium. Reagent, Quantity (for 1 L),

Fungus and bacteria inoculation & incubation; Fungi were inoculated in the center of the plate and incubated at 28°C. Bacterial isolates were inoculated along the side of plates incubated at 28°C

Antifungal activity: Suppression of fungal growth

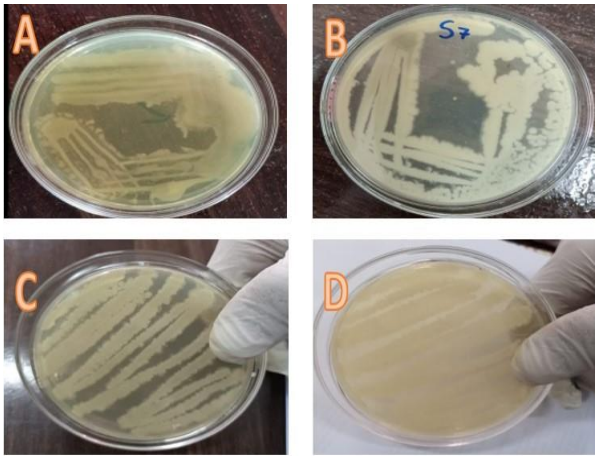
was visualized. Inhibition will be shown either through the discoloration and stress growth of the fungus. or through a zone of inhibition where no hyphal growth is present.

RESULTS

Bacterial growth on nutrient agar medium: Twenty bacterial strains isolated from *Triticum aestivum* rhizospheric soil were inoculated on the nutrient agar plate. Most of the isolated bacterial strains were white morphologically. All of the isolates were rod-shaped.

Figure 2

(A) S20 Bacterial Isolate (B) S7 Bacterial Isolate (C) S4 Bacterial Isolate (D) S1 Bacterial Isolate



The bacterial strains shown in the above figure were grown on a nutrient agar medium at the incubation temperature of 37°C. The Table below shows all the cultural and microscopical findings of isolated bacterial strains.

Cultural and microscopically identification of isolated bacteria

Table 7

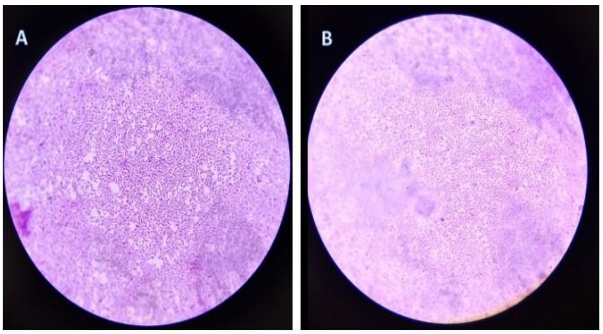
Cultural characteristics of the isolated bacterial strains

Strains	Media	Gram Staining	Cultural Identification	
S1	Nutrient Agar	+	Rod Shaped	White
S2	Nutrient Agar	+	Rod Shaped	White
S3	Nutrient Agar	+	Rod Shaped	White
S4	Nutrient Agar	+	Rod Shaped	White
S5	Nutrient Agar	+	Rod Shaped	White
S6	Nutrient Agar	+	Rod Shaped	Mucoid
S7	Nutrient Agar	+	Rod Shaped	White
S8	Nutrient Agar	+	Rod Shaped	White
S9	Nutrient Agar	+	Rod Shaped	White
S10	Nutrient Agar	+	Rod Shaped	White
S11	Nutrient Agar	+	Rod Shaped	White
S12	Nutrient Agar	+	Rod Shaped	White
S13	Nutrient Agar	+	Rod Shaped	White
S14	Nutrient Agar	+	Rod Shaped	White
S15	Nutrient Agar	+	Rod Shaped	White
S16	Nutrient Agar	+	Rod Shaped	White
S17	Nutrient Agar	–	Rod Shaped	Greenish
S18	Nutrient Agar	–	Rod Shaped	White
S19	Nutrient Agar	–	Rod Shaped	White
S20	Nutrient Agar	–	Rod Shaped	White

Gram staining: Out of the 20 isolated bacterial strains, only 16 were positive for gram stain, while 4 were gram negative. All of the isolates were rods shaped bacilli.

Figure 3

(A) Gram Positive Rod-shaped S3 (B) Gram-negative Rod-shaped S17



The above figure shows the Gram's staining microscopic view of bacterial strains. Figure A represents the Gram-positive rods of the **S3** isolated bacterial strain, while Figure B represents the Gram-negative rods of the **S17** isolated bacterial strain.

Biochemical Test Results

Catalase and Citrate Test: Bacteria colony from each strain (S1-S20) was picked up with wire loop and placed on glass slide. A drop of Hydrogen per oxide was added to slide and bubble formation or absence were noted.

Figure 4

(A) Positive Catalase Test of S1 and S2 (B & C) Positive Citrate Test for S10 and S11

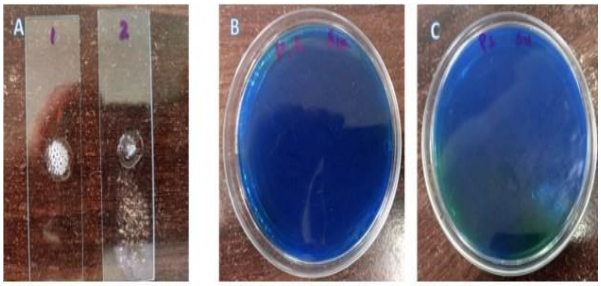
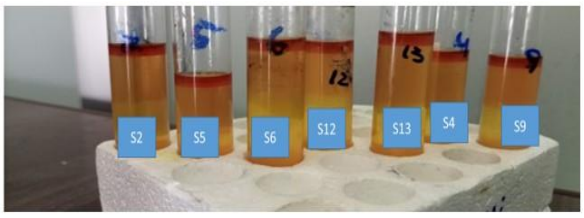


Figure A represents the positive catalase test as evident by formation of oxygen bubbles. A catalase negative will have no bubbles formation. Figure B and C represents the positive citrate test. Both S10 and S11 strains of bacteria was citrate positive as shown. They changed the color of media "from green to blue" in which they were grown (Simon's citrate agar). All of the 20 isolated bacterial strains were positive for citrate utilization test as they all changed the color of media from green to blue.

Indole test: All of the 20 isolated strains of bacteria were incubated on tryptophan soya broth for the 24 hours for the production indole from the decomposition of tryptophan by the action of chain of enzymes.

Figure 5

Indole test is positive for S2, S4, S5, S6, S12, S13 and S9 Bacterial isolates



Out of 20 isolated bacterial strains, only 7 strains (S2, 4, 5, 6, 9, 12, and 13) were positive for indole. Kovac's reagent were added to 24 hours inoculated bacteria, the color changed from yellow to cherry red indicates positive indole production test while no color change remains negative.

Voges–proskauer (VP) test: This test was used to check the ability of microorganism to produce acetymethy carbinol. It will further convert to diacetyl in presence of alpha-napthol.

Figure 6

Shows positive Voges-Proskauer test results for S1-S6 Isolated bacterial samples

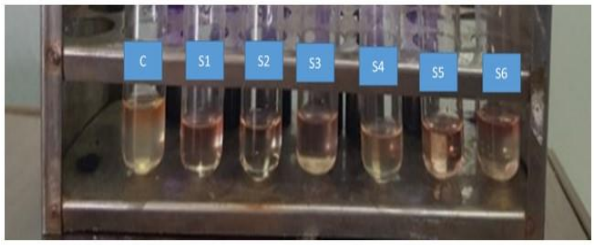


Figure represent the positive VP results for (S1, 2, 3, 4, 5, 6). All of strains except S17, S18, S19, and S20 were positive. VP test is important in biochemical identification of bacteria. Barritt 's reagent A (Alpha-Naphthol "5%" 50 gm, Absolute Ethanol 1000 ml) and Barritt's reagent B (Potassium Hydroxide 400 gm, Deionized Water 1000 ml) were used for performing VP test. Pink color production was taken as positive VP test while no color change represents negative Voges-Proskaur test.

Methyl red test: This test was performed to check the microorganism's ability to produce acid e.g., lactic acid, acetic acid etc.,

Figure 7

All of the bacterial isolates from S1-S20 were negative for Methyl red test

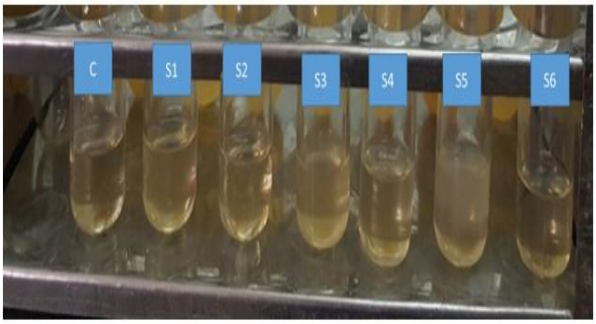


Figure shows negative result for isolated bacterial strains (S1, S2, S3, S4, S5, and S6). All of the 20 isolated bacterial strains were negative for methyl red test.

Biochemical test results

Table 8

Biochemical Test results of 20 isolated Bacterial Strains

Strains	Indole	Catalase	Methyl Red	VP Test	Citrate	Gram Stain
S1	–	+	–	+	+	+
S2	+	+	–	+	+	+
S3	–	+	–	+	+	+
S4	+	+	–	+	+	+
S5	+	+	–	+	+	+
S6	+	+	–	+	+	+
S7	–	+	–	+	+	+
S8	–	+	–	+	+	+
S9	+	+	–	+	+	+
S10	–	+	–	+	+	+
S11	–	+	–	+	+	+
S12	+	+	–	+	+	+
S13	+	+	–	+	+	+
S14	–	+	–	+	+	+
S15	–	+	–	+	+	+
S16	–	+	–	+	+	+
S17	–	+	–	–	+	–
S18	–	+	–	–	+	–
S19	–	+	–	–	+	–
S20	–	+	–	–	+	–

Confirmation of Clinical Strains

Polymerase chain reaction: PCR

PCR results of S1 and S3 Bacterial isolates

Figure 8

PCR results to get expected size bands of 592bp for the amplification of 16s ribosomal RNA sequence

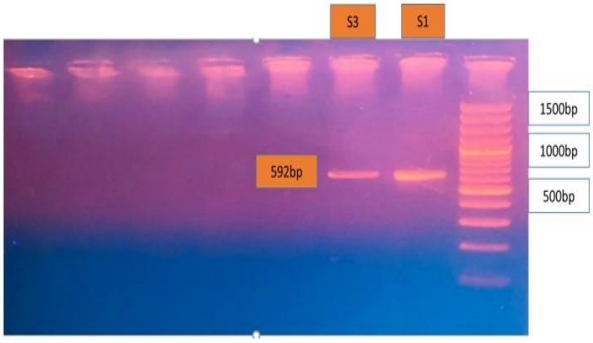


Figure represent the PCR result in gel documentation system for S3 isolated bacterial strain.1500bp ladder was used and 592bp product size bands for S3 and S1 isolated bacterial strains visualized under UV.

PCR Results of S17-S20 Bacterial Isolates

Figure 9

PCR results to get expected size bands of 618bp for the amplification of 16s ribosomal RNA sequence

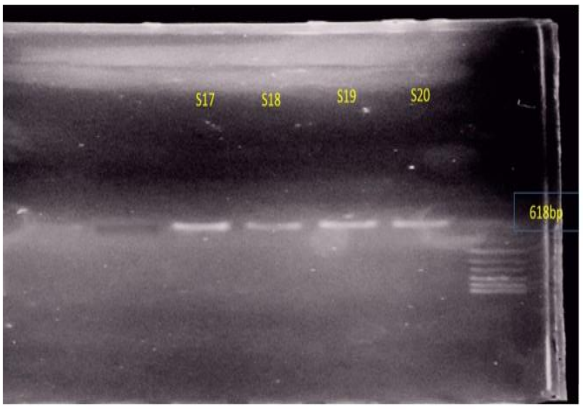


Figure represent the PCR result in gel documentation system for S17 and S18 isolated bacterial strain.1500bp ladder was used and 618bp product size bands for S17 and S18 isolated bacterial strains visualized under UV.

SEQUENCING RESULTS

FASTA Sequence of S18

- CACTGGAACTGAGACACGGTCCAGACT CCTACGGGAGGCAGCAGTGGGGAATA TTGGACAATGGGCGAAAGCCTGATCCA GCCATGCCGCGTGTGTTAAGAAGGTCT TCGGGTTGTAAAGCACTTTAAGTTGGG AGGAAGGGCATTAACCTAATACGTTAG TGTTTTGACGTTACCGACAGAATAAGC ACCGGCTAACTCTGTGCCAGCAGCCGC GGTAATACAGAGGGTGCAAGCGTTAA TCGGAATTACTGGGCGTAAAGCGCGCG TAGGTGGTTTGTTAAGTTGGATGTGAA ATCCCCGGGCTCAACCTGGGAACTGCA TTCAAAACTGACTGACTAGAGTATGGT AGAGGGTGGTGGAATTTCCTGTGTAGC GGTGAAATGCGCAGATATAGGAAGGA ACACCAGTGGCGAAGGCGACCACCTG GACTAATACTGACACTGAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAACGAT GTCAACTAGCCGTTGGGAGCCTTGAGC TCTTAGTGGCGCAGCTAACGCATTAAG TTGACCGCCTGGGGAGTACGGCCGCAA GGTTAAAACTCAAATGAATTGACGGG GGCC

FASTA Sequence of S3

- CAGATGGGTGAGTAACACGTGGGTAA CCTGCCAAGACTGGGATAAGGGGGAT ACCGGATGGTTGTTTGAACCGCATGGT TCAAACATAAAAGGTGGCTTCGGCTAC CACTTACAGATGGAGCGGCGCATTAGC TAGTTGGTGAGGTAACGACCCCGACCT GAGAGGGTGATCGGCCACACTGGGAC TGAGACACGCCTACGGGAGGCAGCAG TAGGGAATCTTCCGCAATGGACGAAA GTCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTTTTCGGATCGTAATCTGT TGTTAGGGAAGAACAAGTACCGTTCGA ATGGTACCTTGACGGTACTAACCAGAA AGCCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGCGTTGTCCG GATATTGGGGCGTAAAGGGCTCGCGG AACTTGAGTGCAGAAGAGGAGAGTGG AATTCCACGTGTAGGTGAAATGCGTAG AGAARGAACAYCAGTGGCGAAGGCGA CTCTCTGGTCTGTACCTGACGCTGAGA GCGAGTGGGGAGCGACAGRATTAGAT ACCCTGGTAGTGCAACGCCGTAACGAT GGTAAGTC

Sequence similarity on NCBI blast: Sequence similarity was checked on NCBI blast. The result obtained from NCBI was showed below. The sequence similarity was 94% for *Pseudomonas fluorescens and* 99% for *Bacillus subtilis.*

Figure 10

Sequence Homology of Pseudomonas fluorescens strain with previously reported sequences on NCBI

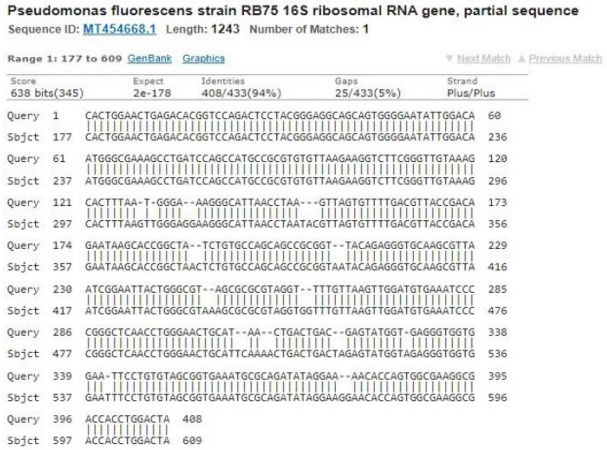
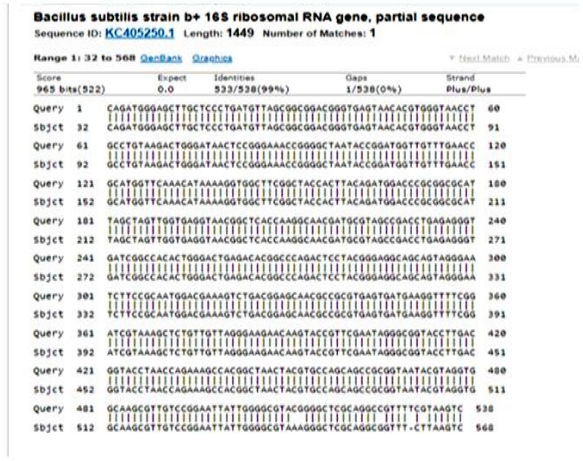


Figure 11

Sequence Homology of Bacillus subtilis strain with previously reported sequences on NCBI

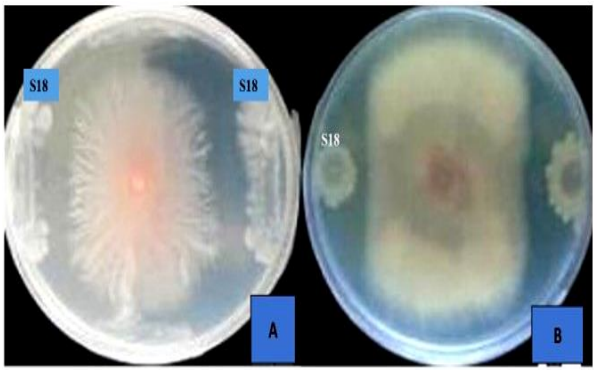


Antifungal activity of *Bacillus subtilis* and *Pseudomonas fluorescens*:

Antifungal activity of *Pseudomonas fluorescens* against *Fusarium oxysporum:* Bacterial strain S18 identified as *Pseudomonas fluorescens* was placed on the edge on plate and the fungus *Fusarium oxysporum* was placed in the middle.

Figure 12

Biocontrol by Pseudomonas fluorescens against Fusarium oxysporum (A) & (B)Fusarium oxysporum in the center and Pseudomonas fluorescens on sides of plates inhibiting the growth

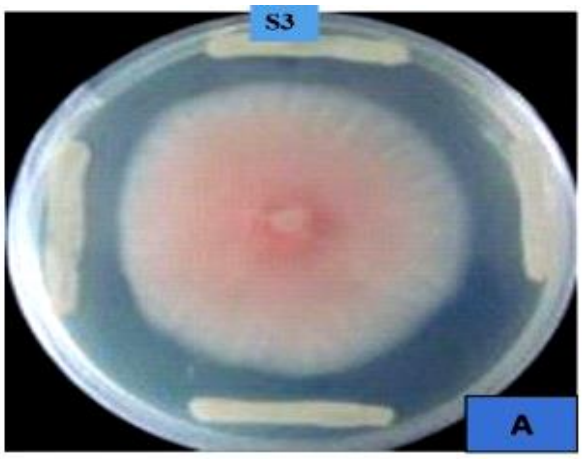


The growth of fungus was suppressed by the *Pseudomonas fluorescens.* This result showed the inhibition of *Fusarium oxysporum* by *Pseudomonas fluorescens*.

Antifungal activity of *Bacillus subtilis* against *Fusarium oxysporum*

Figure 13

Biocontrol by Bacillus subtilis against Fusarium oxysporum (A) Fusarium oxysporum in the center and Bacillus subtilis on sides of plates inhibiting the growth

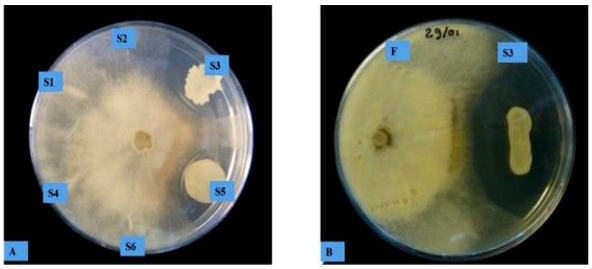


Fusarium oxysporum was grown in the middle of plate containing potato dextrose agar and 0.5% yeast extract "which promotes the growth of bacteria". Figure shows the successful suppression of *Fusarium oxysporum* growth by *Bacillus subtilis.*

Biocontrol by *Bacillus subtilis* against *Botrytis cinerea* (Antifungal Activity)

Figure 14

(A*) Bacillus subtilis* against *Botrytis cinerea* in multiculture (B) *Bacillus subtilis* against *Botrytis cinerea*



In figure (A) *Botrytis cinerea* was placed in center while isolated bacterial strains from (S1-S6) were inoculated on the sides. Bacterial strains S3 and S5 showed inhibition of *Botrytis cinerea* fungus. The S3 strain was later identified as *Bacillus subtilis.* Figure (B) represents the *Bacillus subtilis* (S3) on one side while *Botrytis cinerea* fungus on the other side and grow suppression was visualized.

Biocontrol activity by *Bacillus subtilis* and *Pseudomonas fluorescens:*

Table 9

Biocontrol activity by Rhizospheric bacteria

Bacteria	Fungal pathogen	Resistant/Susceptible
Bacillus subtilis	*Fusarium oxysporum*	Susceptible
Pseudomonas fluorescens	*Fusarium oxysporum*	Susceptible
Bacillus subtilis	*Botrytis cinerea*	Susceptible
Pseudomonas fluorescens	*Botrytis cinerea*	Resistant

DISCUSSION

The present research only shows antifungal activity against Bacillus subtilis and Pseudomonas fluorescens against Fusarium oxysporum. The Bacillus subtilis also showed antifungal activity against Botrytis cinerea. This result follows (Karimi et al., 2012).

Several other researchers also evaluated the antagonistic activity of different bacteria against fungal pathogens. (Karimi *et al*., 2012). They demonstrated negative effects of six *Pseudomonas* isolates and six *Bacillus* isolates from the chickpea rhizosphere that were tested as possible biocontrol agents against *Fusarium oxysporum* f. sp. ciceros

in vitro and *in vivo*. *In vitro* and *in vivo* evaluations of the antagonistic effects of six isolates each of the *Pseudomonas* and *Bacillus* genera against *Fusarium oxysporum* f. sp. ciceris as possible biocontrol agents. Tests for fungus inhibition were run using a plate assay. Cyanide hydrogen, Protease, indole acetic acid, siderophore, antifungal volatile, and extracellular chemical synthesis were examined in each isolate. According to their strong *in vitro* antagonistic efficacy, which was demonstrated as inhibition zones in the dual-culture experiment, twelve isolates were chosen. *Bacillus subtilis* (B1, B6, B28, B40, B99, and B108), *Pseudomonas putida* (P9 and P10), and *P. aeuroginosa* were recognised as the phenotypic characteristics of a few isolates (P11, P12, P66 and P112). Different bacterial isolates have different capacities for producing cyanide hydrogen, siderophore, protease, and indole acetic acid (IAA). Under greenhouse circumstances, bacterial strains' biocontrol effectiveness and ability to promote plant growth were assessed. *P. aeuroginosa* (P10 and P12), *B. subtilis* (B1, B6, B28, and B99), and *P. aeuroginosa* (P12 and B28) all offered better control (P = 0.05) than untreated control (15.8-44.8%) in seed treatment and soil-inoculation, respectively. In both studies, the B28, P12, and P112 isolates considerably outgrew the untreated control in terms of plant height and fresh and dry weight. Their findings suggest that PGPR enhances this plant's growth factors and can aid in the biocontrol of pathogens.

(Mardanova *et al*., 2016) isolated *Bacillus subtilis* GM2 and GM5 bacterial strains from potato root rhizosphere soil tested in vitro and in vivo for possible antagonistic activity against fungal infections. The potential antagonistic activity against fungal pathogens of *Bacillus* strains isolated from the rhizosphere soil of potato roots was assessed *in vitro* and *in vivo*. By analysing their 16S rRNA and GyrB genes, two bacterial isolates were recognized as novel *Bacillus subtilis* strains and given the labels GM2 and GM5, respectively. The capability of some strains to prevent the growth of several phytopathogenic fungi served as a defining characteristic. It was demonstrated that the GM5 strain outperformed the GM2 strain at preventing the development of phytopathogenic fungi. Both cultures may generate many hydrolytic enzymes and antibiotic compounds (ammonia and HCN). The GM2 strain also generated siderophores. BacA, srfA, ItuC and bmyB,, four genes that encode antimicrobial peptides, were found in the genome of the GM2 strain. The antimicrobial peptide genes srfA and fenD were present in the GM5 genome. GM5 strain's purified lipopeptide fraction, but not GM2 strains, was able to restrain the spread of *Fusarium solani* in the plate assay. Additionally, the GM2 strain of *Bacillus subtilis* aided in the development of wheat, but only the GM5 strain could shield wheat seedlings from *Fusarium oxysporum* infection (Validov, 2007).*Pseudomonas putida* strain PCL1760 against different fungal pathogens *(Fusarium oxysporum f.sp. radicis-lycopersici*) of the foot and root rot of tomatoes.

(Chen *et al*., 2022) showed that one of the most significant PGPRs is a Bacillus velezensis strain SDTB038 that has biocontrol effects that was isolated and identified in a prior investigation. Whole genome sequencing of strain SDTB038 revealed seven secondary metabolite biosynthesis gene clusters, which account for its biocontrol properties. Results showed that various doses of SDTB038 fermentation broth reduced Fusarium crown and tomato root rot mycelial development. While the effect of 108 CFU/ml SDTB038 concentration on encouraging tomato growth was the most noticeable, strain SDTB038 could produce indole acetic acid and encourage healthy growth of tomatoes.

CONCLUSION

Out of 10 soil samples, 20 bacterial samples were isolated. Sixteen samples were Gram-positive, while 4 samples were Gram-negative. The Biochemical test and molecular identification resulted in the isolation of Bacillus subtilis and Pseudomonas fluorescens in 20 bacterial samples. Sequence identity: The S3 bacterial sample showed 99% identity to Bacillus subtilis, while S18 showed 94% identity to Pseudomonas fluorescens. Bacillus subtilis was a good biocontrol agent against Botrytis cinerea and Fusarium oxysporum. Pseudomonas fluorescens was proved a promising biocontrol agent against Fusarium oxysporum only.

Future Prospects: Further research is required to increase the efficacy of these biocontrol bacteria.

How can these bacteria be used on an industry level to overcome the excess use of pesticides? Development of suitable formulations of these biocontrol agents. Synergistic effects with rhizobacterium should be investigated. These bacteria might control other fungal plant pathogens, which needs to be explored.

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